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Skylar-Scott, Mark A ; Liu, Man-Chi ; Wu, Yuelong ; Yanik, Mehmet Fatih

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DOI: https://doi.org/10.1117/12.2253520

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Mark A. Skylar-Scott, Man-Chi Liu, Yuelong Wu, Mehmet Fatih Yanik, "Multi-photon microfabrication of three-dimensional capillary-scale vascular networks," Proc. SPIE 10115, Advanced Fabrication Technologies for Micro/Nano Optics and Photonics X, 101150L (28 April 2017); doi: 10.1117/12.2253520

SPIE.

Event: SPIE OPTO, 2017, San Francisco, California, United States

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ABSTRACT

Biomimetic models of microvasculature could enable assays of complex cellular behavior at the capillary-level, and enable efficient nutrient perfusion for the maintenance of tissues. However, existing three-dimensional printing methods for generating perfusable microvasculature with have insufficient resolution to recapitulate the microscale geometry of capillaries. Here, we present a collection of multiphoton microfabrication methods that enable the production of precise, three-dimensional, branched microvascular networks in collagen. When endothelial cells are added to the channels, they form perfusable lumens with diameters as small as 10 μm. Using a similar photochemistry, we also demonstrate the micropatterning of proteins embedded in microfabricated collagen scaffolds, producing hybrid scaffolds with both defined microarchitecture with integrated gradients of chemical cues. We provide examples for how these hybrid microfabricated scaffolds could be used in angiogenesis and cell homing assays. Finally, we describe a new method for increasing the micropatterning speed by synchronous laser and stage scanning. Using these technologies, we are working towards large-scale (>1 cm), high resolution (~1 μm) scaffolds with both microarchitecture and embedded protein cues, with applications in three-dimensional assays of cellular behavior.
Keywords: 3D printing, capillary networks, microvasculature, hybrid scaffolds, cell homing, biotin-4-fluorescein

1. INTRODUCTION

Decellularized organs, stripped of their cells, retain an exquisitely microarchitected extracellular matrix (ECM) scaffold, replete with microchannels where capillaries or ducts once lay, and heterogeneous three-dimensional (3D) patterns of basement membrane, connective fibrillar tissue, and growth factors[1,2]. With the goal of *de novo* fabrication of tissues, 3D bioprinting is a powerful approach for engineering complex, heterogeneous 3D cellular environments, with potential applications in developmental assays and drug screening[3–5]. Furthermore, as large-scale tissues become necrotic without a functional vasculature, several recent methods for bioprinting vascularized tissues are promising for therapeutic-scale tissue engineering[6–8]. However, existing bioprinting approaches fail to recapitulate defined structures with the resolution of capillary beds – where most nutrient and gas exchange occurs.

In contrast to bioprinting, several direct laser-writing methods for microfabricating endothelial-lined microvascular networks are approaching capillary-scales. Laser-driven photo-ablation of PEG or collagen scaffolds have achieved microvascular channels that are approximately 50 μm in diameter[9]. Recently we demonstrated a multi-photon photolithography (MPP) approach for fabricating endothelial-lined branched vascular networks in collagen type I with lumen diameters as small as 20 μm - approaching that of capillaries[10]. We also described an MPP approach for incorporating micropatterns of proteins embedded inside the microfabricated collagen scaffold, generating scaffolds with both defined microarchitecture and chemical cues. Using these hybrid scaffolds, we demonstrated leukocyte homing to specific 3D locations in the vascular network by patterning P-selectin.

However, our hybrid MPP approach had several key limitations. First, the protein cues we printed in the microarchitected collagen were quasi-planar in nature, and did not exhibit arbitrary gradients. Second, the collagen scaffold required acid-solubilization to develop the microarchitecture, hindering its biocompatibility with embedded cells. Third, the leukocyte homing was driven by patterned P-selectin on the walls of a microvascular collagen scaffold,

Advanced Fabrication Technologies for Micro/Nano Optics and Photonics X, edited by Georg von Freymann, Winston V. Schoenfeld, Raymond C. Rumpf, Proc. of SPIE Vol. 10115, 101150L
 · doi: 10.1117/12.2253520

whereas in the *in vivo* environment, P-selectin is presented by activated endothelial cells. Finally, the microfabrication method was too slow to produce patterns at centimeter-scales.

Here, we present several upgrades to our system, and describe several alternative approaches to address each of these issues. First, we now demonstrate the ability to read in analog (i.e. gradient) voxel datasets to generate arbitrary 3D gradients inside arbitrary 3D geometries, generating intricate hybrid microarchitected and micropatterned scaffolds. Next, we describe a method for the rapid microfabrication of 3D collagen scaffolds by fluorescein-driven ablation of collagen, overcoming the requirement of an acid solubilization step, and potentially enabling the microfabrication of cellular scaffolds. In contrast to the photoablation approach of Lutolf and colleagues[9], the presence of fluorescein enables us to produce microvascular networks at scan-speeds more than three orders of magnitude faster. To demonstrate a more *in vivo* relevant leukocyte homing system, we demonstrate the ability of activated endothelium in manufactured microcapillaries to elucidate rolling of leukemia cells when perfused through the lumens. Finally, to address the issue of scalability, we describe a new MPP approach that enables continuous scanning by synchronizing galvanometer mirror scanning and stage translation. Using this approach, we densely micropatterned a 1 cm^2 fibrin scaffold in approximately one hour.

2. OPTICAL SETUP

A multi-photon photopatterning (MPP) setup was constructed using a *MAI-TAI* femtosecond laser tuned to 780 nm emission. The laser was scanned via a pair of *x-y* galvanometer scanning mirrors (Cambridge Technology, Bedford MA), and a piezo objective mount provided *z*-focus control (Physik Instrumente GmbH, Karlsruhe, Germany) (*Fig. 1*). The sample was placed on a servo-motor *x-y* translatable stage (Prior Scientific, Rockland MA). A pair of scanning mirrors were used to image the scanning mirrors onto the back aperture of a 40X, 0.9NA air-immersion objective, and to expand the beam to ensure the beam diameter exceeds that of the back aperture for maximal photopatterning resolution. A short-pass dichroic mirror (Edmund Optics, Barrington NJ) was selected to enable a small percentage (< 1%) of light reflected from the sample to transmit to the CMOS camera for ease of alignment. An electro-optic modulator (EOM), with 8 MHz bandwidth is used to modulate the laser power. In this setup, a single National Instruments 6259 Board, equipped with four analog outputs, is used to control the *x* and *y* scanning mirrors, the EOM transmission, and the piezo mount for the objective. The scan mirrors can be operated at close to their resonant frequency for high-speed photopatterning applications.

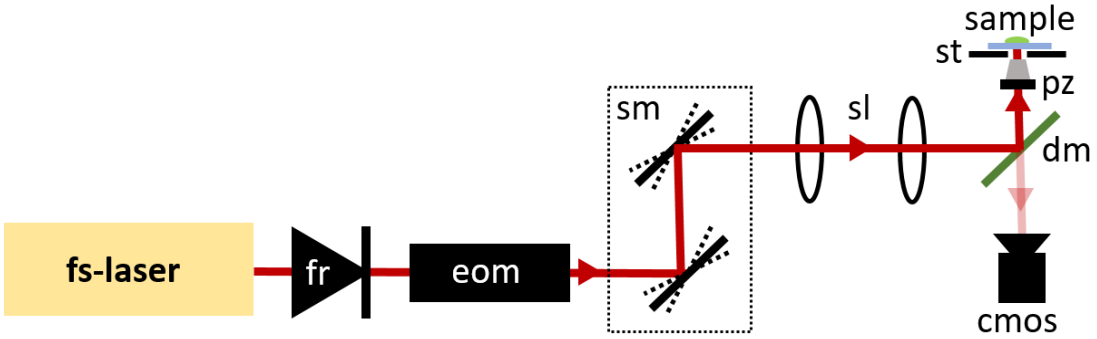

Figure 1. Optical setup of multi-photon microfabrication system. Fs-laser = femtosecond-pulsed laser. Fr = faraday rotor. Eom = electro-optic modulator. Sm = *x-y* galvanometer scanning mirrors. Sl = beam expanding scan lenses. Pz = objective piezo mount for *z* focus control. Dm = shortpass dichroic mirror. Cmos = CMOS alignment camera. St = *x-y* servo-motor stage with encoder.

3. RESULTS AND DISCUSSION

3.1 Multi-photon 3D micropatterning of protein gradients within collagen scaffolds

To build upon our previous work in generating 2D graded patterns embedded inside a bulk hydrogel, here, we used the MPP setup described above, with a new data interpreter and printer controller, to produce fully 3D graded protein patterns. As a demonstration, we used an MRI voxel dataset, as this constitutes an analog (i.e. non-binary) 3D array of data to test the ability to generate fully 3D protein patterns with gradients (Fig. 2). To form 3D patterns of protein, a

molten gelatin methacrylate (gelMA) hydrogel is cast, and cured under UV light (Fig. 2a, i). Next, biotin-4-fluorescein (B4F) is added, and is allowed to diffuse throughout the hydrogel. The laser is then scanned in *x*, *y*, and *z* to raster a 3D pattern of B4F, layer-by-layer (Fig. 2a, ii). Finally, the unbound B4F is rinsed out, and fluorescently labelled streptavidin is added, whereupon it binds B4F to generate a protein pattern within the hydrogel (Fig. 2a, iii). To prepare a voxel data set for printing, 3D MRI data is unpackaged into 2D axial slices, passed to an interpreter that relays pixel brightness to the required laser power for generating fluorescent streptavidin patterns with equivalent brightness, and is uploaded to the PCIe National Instruments card ready for photopatterning (Fig. 2b). The resulting 3D streptavidin patterns, embedded within the gelMA scaffold demonstrate sub-micron resolution and smooth gradients (Fig. 2c). The resultant full voxel data set of fluorescent streptavidin, imaged under two-photon microscopy, can be rendered back into the original 3D MRI data (Fig. 2d). This ability to generate fully 3D gradients of protein can enable myriad applications for cell assays of migration, polarization, and maturation. Here, we used streptavidin as the fluorescent protein, but any biotinylated protein can subsequently be added to bind to the available streptavidin sites to encode 3D function into the hydrogel.

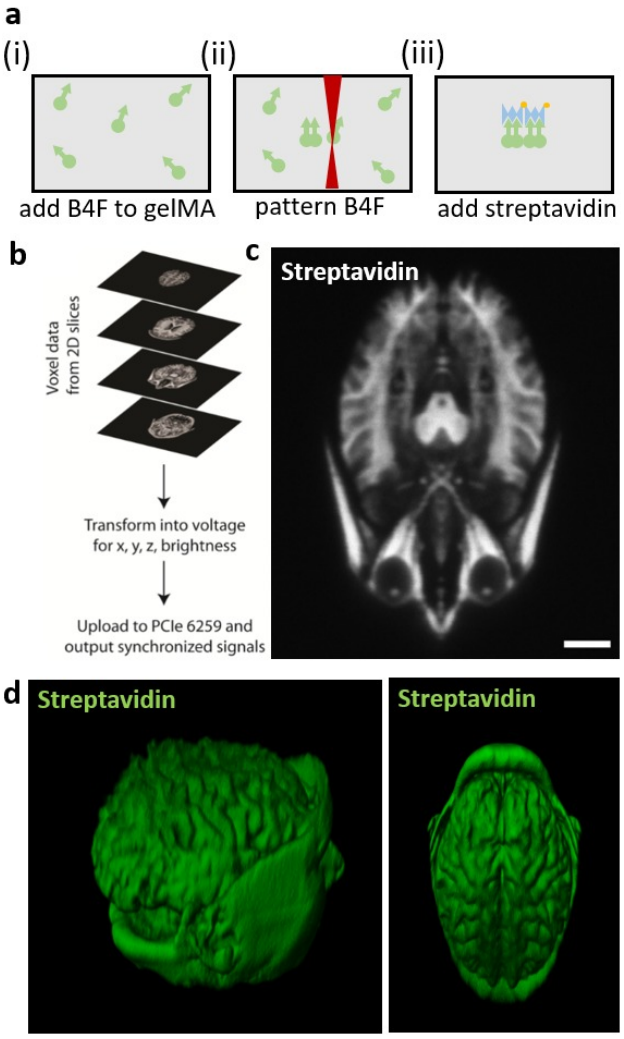

Figure 2. 3D gradients of fluorescently labeled protein inside a bulk gelMA scaffold. (a) Method of producing protein gradients: (i) B4F is added to the gelMA hydrogel, and (ii) is patterned by a laser scanning in the *x*, *y*, and *z*-axes. (iii) Unbound B4F is removed and fluorescently labelled streptavidin is added to bind to the bound B4F patterns. (b) The data interpreter reads 3D MRI voxel datasets, and converts the pixel brightness into the required laser power – i.e. EOM voltage - to generate a set of instructions to print a 3D fluorescent dataset. (c) An example streptavidin pattern that results from the patterning process. Scale bar = 10 μm. (d) 3D renderings of the fluorescent streptavidin patterns, shown in oblique and top-down views reconstitute the original MRI data, with a clearly resolvable brain complete with gyri and sulci.

3.2 Multi-photon microfabrication of collagen scaffolds

In addition to the ability to pattern proteins into the bulk of a 3D scaffold, as described above, our MPP setup can be used to microfabricate the scaffold itself. Collagen type I is a major component of the extracellular matrix, exhibiting excellent biocompatibility, cell adhesion, and degradability properties, and thus has myriad applications as a tissue engineering scaffold. We have developed two orthogonal and complementary approaches for the 3D laser microfabrication of collagen scaffolds, both of which are driven by the multi-photon excitation of fluorescein (Fig. 2). The first method, which we described previously, uses the change in acid-solubility that is imparted to the collagen upon exposure to the laser in the presence of fluorescein (Fig. 3a). Here, we introduce a different method of collagen scaffold manufacture that uses higher laser powers to directly ablate the collagen to produce vascular channels. Importantly, the laser exposure that is required for ablation is dramatically reduced in the presence of soluble fluorescein, enabling scan speeds of up to 350 mm/s. We hypothesize that this is due to enhanced photoabsorption and dye-ionization at the focal point. Supportive of the role of fluorescein in the ablation process, the speed at which ablation occurs is dependent on the concentration of fluorescein in the collagen scaffold (Fig. 3b, ii). This approach builds on previous methods for laser etching of 2D sheets of collagen[11] in that it can produce fully 3D microarchitected scaffolds, and the use of a fluorescein dye dramatically enhances the speed by > 3 orders of magnitude of collagen laser ablation over non-dye incorporating methods[9].

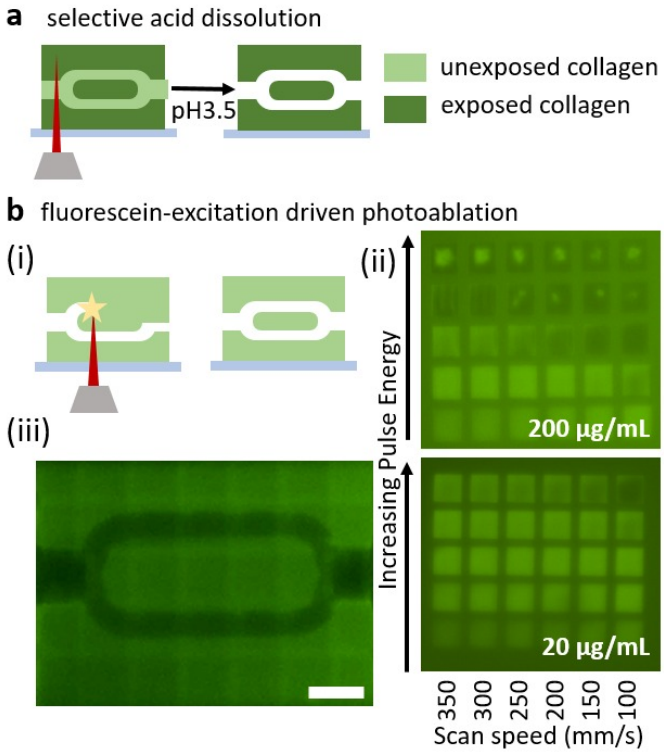

Figure 3. Two methods for multiphoton microfabrication of vascular collagen channels. (a) Using selective acid dissolution, a 3D volume of a fluorescein-infused collagen gel is exposed to a scanning focused femtosecond-pulsed laser. Upon irradiation, the collagen is rendered acid insoluble. Thus, addition of acetic acid (pH 3.5) dissolves the unexposed collagen regions. More details on this approach can be found in our previous work[10]. (b) (i) In fluorescein-excitation driven photoablation, a volume of the fluorescein-infused collagen gel is exposed to a high-power scanning focused femtosecond-pulsed laser, ablating the collagen, rendering a channel that is inscribed in the gel. (ii) The speed, power, and exposure at which the ablation occurs depends upon the concentration of fluorescein solution used – here comparing 200 µg/mL and 20 µg/mL gels. (iii) An example microvascular channel generated in a collagen gel. Scale bar = 100 µm.

3.3 Multi-photon microfabrication of collagen scaffolds

We previously described a method for combining the MPP microfabrication of a collagen scaffold (Fig. 3) with the ability to pattern proteins inside the scaffold (Fig. 2). Here, we greatly enhance the complexity of this work by using the 3D voxel dataset interpreter to generate truly complex 3D collagen scaffold microarchitectures containing arbitrary gradients of streptavidin. To perform this hybrid printing method, we first infuse a collagen gel with fluorescein, and pattern a first volume – this will become the collagen scaffold (Fig. 4a). Next, we infuse the gel with B4F and pattern a second volume – this volume will become the regions of the collagen scaffold that are decorated with fluorescent streptavidin. After micropatterning, the gel is developed in acetic acid (pH 3.5) and streptavidin is added to bind to the patterned B4F.

Here, we use a complex mathematically defined 3D knot-like model to generate a microarchitected collagen scaffold (Fig. 4b). The resulting scaffold exhibits exquisite 3D structure on multiple levels: the microarchitecture of the scaffold, and the embedded gradients of streptavidin. We note that arbitrary gradients of streptavidin can immediately enable gradients of any functional cue by addition of biotinylated proteins, peptides, RNA, DNA, or small molecules to bind to the available streptavidin sites. This process is fast on a per-unit basis, requiring just 10 seconds of laser scanning time to define both the microarchitecture and B4F cues. Note that this is a per-unit time; there is a significant fixed time (> 1 hr) associated with collagen scaffold gelation, fluorescein and B4F diffusion, acid dissolution, and streptavidin diffusion.

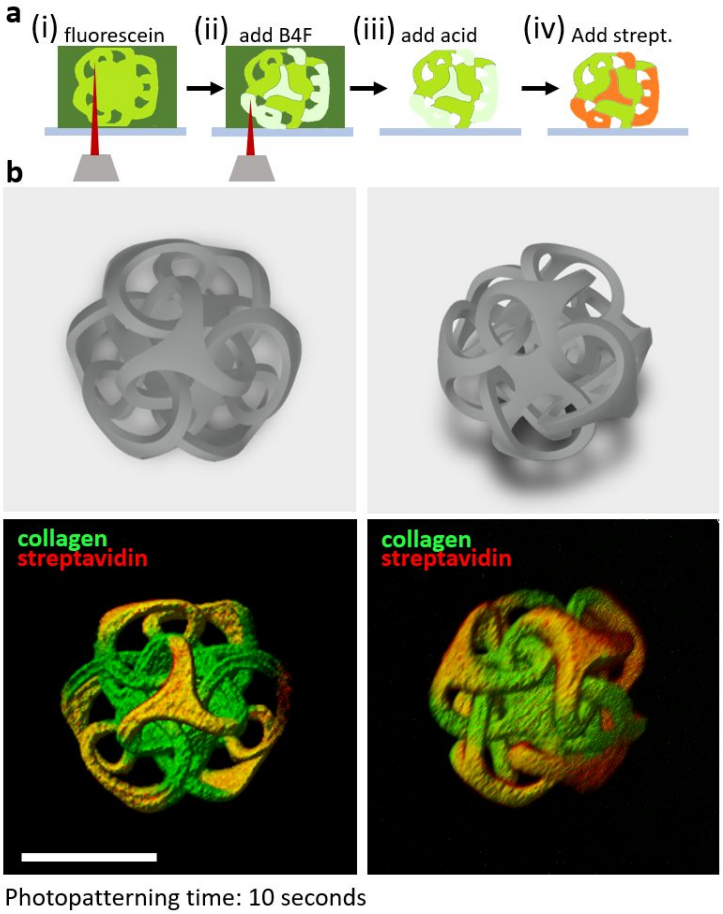

Figure 4. Hybrid MPP of collagen scaffold microarchitecture containing embedded micropatterned gradients of streptavidin. (a) Hybrid scaffold microfabrication process, whereby (i) a fluorescein infused collagen gel (dark-green) is exposed to a rastering fs-laser (mid-green), (ii) B4F is allowed to diffuse into the scaffold and a second volume is exposed (light green). (iii) The unexposed scaffold (dark green) is dissolved via acid adition, and (iv) streptavidin (orange) is added to bind to the patterned B4F. (b) 3D knot-like models used for microfabrication, and the resulting 3D renderings of microfabricated collagen knots containing patterned regions of streptavidin. Fabrication time = 10 seconds. Scale bar = 50 μm.

3.4 Microfabrication of capillary-scale perfusable vasculature

To demonstrate the biological applications of microarchitected collagen scaffolds, we designed 3D models of vascular networks, and manufactured capillary scale blood vessels into collagen scaffolds (Fig. 5). The scaffolds are manufactured in a microfluidic channel, as we previously described[10]. After scaffold fabrication, endothelial cells are flowed into the networks via the microfluidic device, and flow is ceased to allow the cells to adhere to the walls (Fig. 5a). Crucially, the collagen scaffold must be glutaraldehyde crosslinked prior to addition of the cells to prevent rapid scaffold degradation from occurring. After the cells adhere migrate along the collagen channels, they form lumenized microcapillaries. At their narrowest, these microcapillaries have a lumen diameter of ~ 10 μm, and consist of a single vacuole-containing endothelial cell that wraps around the channel circumference (Fig. 5b inset).

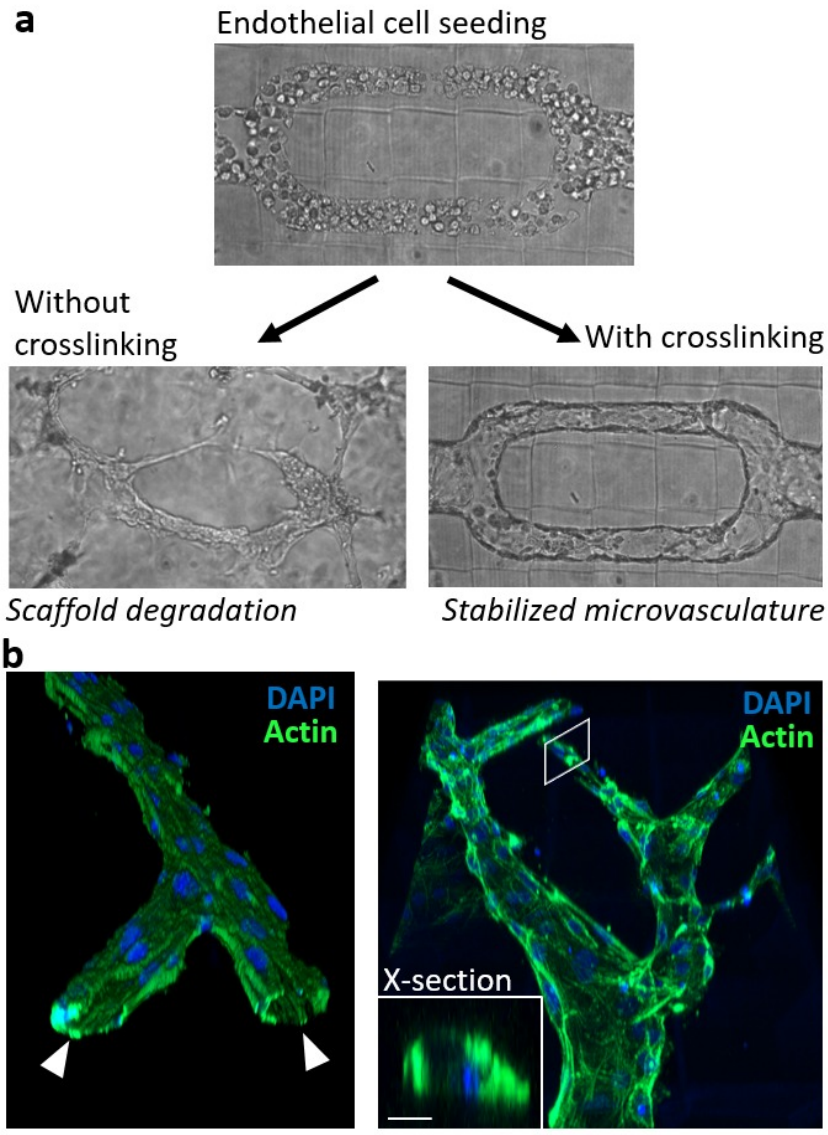

Figure 5. Microfabricated capillary networks formed in collagen scaffolds containing branched 3D channels. (a) After development in acid, treatment of collagen scaffolds with glutaraldehyde prevents premature degradation of the scaffold. (b) Microfabricated branched capillaries with patent lumens (arrowheads). Scale bar on inset = 10 μm.

3.5 Example assays on microfabricated capillary networks

By combining the microcapillary microfabrication with the hybrid approach described in Fig. 4, we are conducting assays for probing cell migration, angiogenesis, and cell homing in microfabricated capillary networks (Fig. 5). By

patterning regions adjacent to channels with gradients of cues such as VEGF (Fig. 5a), we aim to direct angiogenesis to combine our microfabricated approach of generating microvasculature with self-assembled microvasculature. In our previous work, we studied adhesion and rolling of leukocytes on acellular microchannels, patterned with P-selectin. Now, we are beginning to employ pyrogens or cytokines, such as lipopolysaccharides or interleukin-1 to conduct assays of leukocyte adhesion and rolling on branched, endothelial lined microvascularized channels. Via these assays, we are aim to study cellular behaviors relevant to vascular development, inflammation, atherosclerosis, and cancer metastasis at the microcapillary scale.

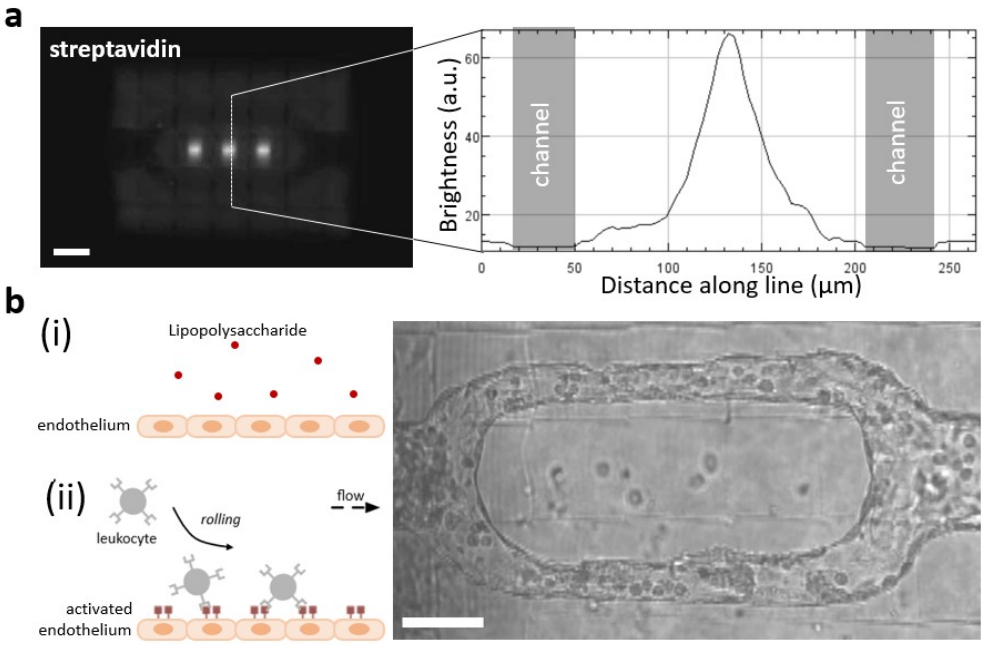

Figure 6. Example assays that can be performed using a hybrid MPP approach. (a) Microvascular collagen scaffolds are flanked by 3D micropatterned protein gradients. The intention in this hybrid scaffold assay is to study angiogenic potential of different protein gradients by studying the interaction of adjacent microvessels with the collagen-bound gradient. Example biotinylated proteins that will be assayed include vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF). The adjacent graph displays the pattern brightness of streptavidin between the two channels.(b) Example assays of cell homing and rolling in microvascular networks. Endothelial cell-lined capillary networks can be activated by exposure to interleukin-1 or lipopolysaccharide. When leukocytes are flowed through the capillaries (spherical cells inside the micovasculature), they adhere and roll along the wall. Scale bar = 100 μm.

3.6 High-speed microfabrication of protein patterns in scaffolds via continuous-scan laser microfabrication

While our MPP approach demonstrates the ability to microfabricate high resolution microcapillary networks, and despite the high rastering speed we use to create our models (up to 400 mm/s), the process still lacks the scalability for therapeutic-scale biofabrication. One significant delay in an *x*-*y* scanning system is that once a field of view is completed, the stage must be translated to a new location before resuming laser scanning.

Thus, we have redesigned our system for high-speed micropatterning, performing 'continuous-scan laser microfabrication' (CSLM) in which the laser scans along the x-axis, while the stage scans continuously in the y-axis. By modulating the laser power, we can continuously generate patterns in a Since the range of the stage motion is >10 cm, this enables continuous pattern formation for long duration. Figure 7 shows the production of a 1 cm^2 checkerboard-patterned area of streptavidin into a fibrin scaffold using CSLM, requiring approximately 1 h of fabrication time. Note that the fibrillar architecture of the fibrin scaffold becomes apparent as the fluorescent streptavidin decorates the fibrin fibrils. In this process, the *x*-axis galvanometer scan range was 100 μm, while the stage was translated along the *y*-axis at a speed of 0.5 mm/s. If this area were to be micropatterned via sequential patterning of 100 μm x 100 μm regions, then the stage would need 10,000 start and stop motions. However, using CSLM, the patterning only stops at the end of each 100 μm strip. Thus, the stage only requires 100 start and stop motions, dramatically reducing patterning down time. If

3D patterns are desired, the *z*-axis piezo can be stepped after the completion of each 1 cm^2 area. Using CSLM, we are exploring therapeutic-scale production of hybrid microfabricated scaffolds.

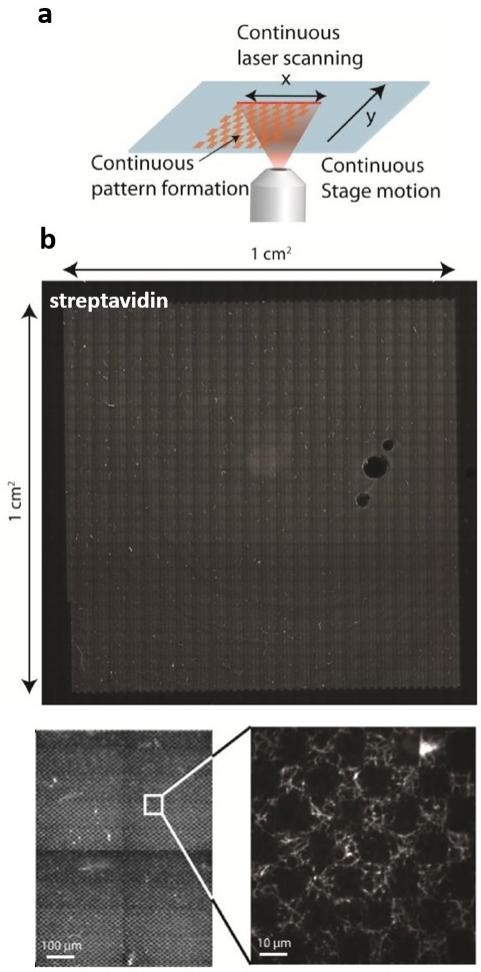

Figure 7. Continuous scan laser lithography enables the rapid micropatterning of 1 cm^2 areas. (a) In CSLM, the laser scans bidirectionally along the *x* axis, while the stage translates continuously in the *y*-axis. By synchronous modulation of laser intensity, micropatterns are continuously produced behind the scanning stage. In this manner, large areas, greater than the scan-range of the galvanometer mirrors, can be constructed in a continuous manner. (b) A 1 cm^2 fibrin scaffold micropatterned with a checkerboard pattern of fluorescent streptavidin using CSLM.

4. CONCLUSIONS

MPP can be used to generate complex, hybrid scaffolds with microarchitecture and embedded patterns of protein cues. We have described several approaches that enhance the complexity, scale, and speed of our approach. First, we demonstrate fully 3D graded patterns of protein cues in scaffolds which could be used for 3D assays of cellular behavior. Next, we describe a new high speed method for microfabricating channels in collagen scaffolds via fluorescein-driven ablation. This approach is more suited to producing microchannels in scaffolds than our previous acid-dissolution approach, where only the sparse, channeled regions require exposure to the laser. We have demonstrated highly complex hybrid collagen scaffolds with intricate knot-like geometries and embedded gradients of proteins. When endothelial cells are added to the microfabricated collagen scaffolds, they form architected, branched microcapillary networks with lumens as small as 10 µm. We describe cell migration and leukocyte adhesion assays that can be undertaken using hybrid MPP. Finally, we demonstrate a rapid method for producing large-scale scaffolds, 1 cm^2 in area, in less than 1 hr. In the future, we aim to further enhance patterning speed through optical, mechanical and photochemical advances to enable capillary-scale tissues to be manufactured at therapeutic scales.

5. EXPERIMENTAL METHODS

5.1 3D micropatterning of proteins in gelMA scaffolds

GelMA was manufactured and lyophilized as previously described[12]. The lyophilized gelatin was reconstituted at 100 mg/mL in PBS, and 5 mg/mL of the photoinitiator Irgacure-2959 was added. The gel was cast between two cover slips, spaced by ~20 μm, and exposed to 5 mW/cm^2 UV light for 2 mins to photo-crosslink the scaffold. The top coverslip was removed, and the scaffold was allowed to equilibrate in a bath of 200 μg/mL solution of B4F (Thermo Fisher Scientific) in PBS. Next, the B4F was exposed to a rastering femtosecond-laser MPP setup, as described above. After exposure, the unbound B4F was removed by rinsing the scaffold in an excess of PBS for > 30 minutes, and a 10 μg/mL streptavidin-Alexa Fluor 555 solution (Thermo Fisher Scientific) in PBS containing 30 mg/mL of solubilized bovine serum albumin (BSA) was added to bind to the B4F. The scaffold was then rinsed with PBS for > 30 minutes to remove unbound streptavidin.

5.2 3D microfabrication of collagen scaffolds

Rat tail collagen type I (Corning) was maintained on ice, and diluted in PBS and neutralized by addition of 1N NaOH to a final concentration of 6 mg/mL. The neutralized, cold collagen was pipetted into a microfluidic channel as previously described[10], and incubated at 37C for gelation to occur. The collagen scaffold was then incubated with a 200 μg/mL solution of fluorescein free acid (Sigma Aldrich) in PBS – prepared by adding a 10 mg/mL stock solution of fluorescein free acid dissolved in DMSO into PBS. To perform laser ablation, the fluorescein-infused scaffold was exposed directly to a high-power rastering fs-laser. Alternatively, to perform collagen manufacture via acid-dissolution, we exposed regions of the channel to the rastering laser, then rinsed the scaffold with 0.1M acetic acid, pH 3.5 for >1 hr.

5.3 Hybrid microfabrication and micropatterning of collagen scaffolds

Collagen scaffolds were prepared as described above, and fluorescein-infused collagen was exposed to a rastering laser at a laser power of ~2 nJ/pulse and a scan speed of 200 mm/s. The fluorescein solution was then carefully replaced with a 200 μg/mL B4F solution without displacing the sample to maintain 3D alignment. A second volume was exposed to a rastering laser. Next, the B4F solution was rinsed with PBS before adding a 0.1M, pH 3.5, acetic acid solution to dissolve the unexposed collagen for >1hr, and rinsed 3 times with fresh 0.1M acetic acid solution to remove dissolved collagen. The developed collagen scaffold was then neutralized by rinsing 3 times with PBS. Next, 10 μg/mL of fluorescent streptavidin in PBS containing 30mg/mL BSA was added to bind to the patterned B4F. Finally, the hybrid scaffold was then rinsed with PBS to remove unbound streptavidin.

5.4 Cell culture, formation of microvasculature, and rolling assays

Human umbilical vein endothelial cells (HUVECs) (Lonza, Basel, Switzerland) are cultured in EGM medium (Lonza) on tissue-culture treated flasks, passaged at 80% confluency using trypsin, and used for up to 9 passages. For forming microvascular channels, EGM containing HUVECs in suspension are added to collagen microchannels at > 10^6 cells mL^{-1}, and are allowed to adhere in an incubator at 37C before adding more medium to each well. HL-60 leukemia cells are cultured in suspension in cell culture flasks with IMDM containing 10% fetal bovine serum. The cells are passaged by pelleting, removing the supernatant, resuspending in 10 mL, and splitting 1:10 into a new culture flask. For rolling assays, endothelial-lined microvasculature were exposed to lipo-polysaccharides at 10 μg/mL for 10 minutes to activate endothelium. 4 h after exposure, HL-60 cells were flowed through the channels to observe rolling.

5.5 High-speed micropatterning of fibrin scaffolds.

Fibrin scaffolds were prepared by dissolving 10 mg fibrinogen into 1 mL of PBS for 1 h at 37C. Care must be taken not to agitate the solution into dissolution is complete. Next, 5 μL of a 100 U/mL solution of thrombin in PBS was added to the fibrin, on ice. The solution was immediately pipetted between two coverslips spaced by ~20 μL and warmed to room temperature and incubated for 10 min to complete gelation. After gelation, the top coverslip is removed and the scaffold is incubated in 200 μg/mL of B4F for 30 mins, and is placed on the stage for printing. The stage is scanned at a constant speed of 0.5 mm/s up and down the *y*-axis across a 1 cm^2 area in a 'snake' pattern, with stage scan-lines spaced parallel to the *x*-axis at 100 μm. Concurrently, the laser is scanned across a 100 μm line at 200 mm/s parallel to the *x*-axis, while the EOM modulates the laser power to generate the patterns.

ACKNOWLEDGEMENTS

The authors would like to thank Bathsheba Grossman for generously providing the 3D 'metatron' model used for Figure 4, and the University of North Carolina for use of its volume data archive for the MRI data presented in Figure 2. M.A.S.-S. was supported via a Martinos Scholarship. This project was funded by an NIH R01 EUREKA Award (R01-NS066352), a Packard Award in Science and Engineering, and an NIH Director's Pioneer Award (DP1-NS082101).

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